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AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent
Application Serial No. 696764 by Human
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by
Ludwig Institute for Cancer Research

STATUTORY DECLARATION

I, Kari Alitalo, a Research Professor of the Finnish Academy of Sciences, at The Molecular/Cancer Biology Laboratory, Biomedicum Helsinki, P.O.B. 63 (Haartmaninkatu 8) 00014 University of Helsinki, Finland, do solemnly and sincerely declare as follows:

I. INTRODUCTION

1.1 In July, 2002, I executed a third statutory declaration (hereinafter referred to as "OKA3" (Opponents, Kari Alitalo, 3rd Declaration)) to provide experimental evidence in support of the opposition filed by Ludwig Institute for Cancer Research ("Ludwig Institute") to the issuance of a patent to Human Genome Sciences, Inc., ("HGS") based on HGS's Australian Patent Application No. 696764 ("the opposed application"). A brief summary of the various declarations that have been filed throughout these proceedings was provided in OKA3 and will not be repeated here.

1.2 I hereby reaffirm my understanding that I have an overriding duty to the Patent Office (and to any Australian Federal Court that should review the Patent Office decision) to provide objective scientific analysis that I believe to be truthful. I hereby affirm that, to the

best of my knowledge and belief, factual statements herein are true and opinion statements herein represent my objective scientific opinion and analysis.

1.3 As described in OKA3, the purpose of my third statutory declaration was to design and perform protein expression experiments that would address any criticisms raised in the second series of declarations filed on behalf of HGS, particularly the second declarations of Dr. Nicholas Hayward (ANH2) and Dr. Stuart Aaronson (ASA2). Further, Ludwig Institute asked me to comment on the data from expression studies performed by Dr. Susan Power and reported in her second declaration (hereinafter "ASP2"), filed at the same time as ANH2 and ASA2.

1.4 The purpose of this declaration, hereinafter referred to as "OKA4", is to supplement the findings in OKA3 with an additional figure that depicts the results of transfection experiments for the Australian Patent Office. The results described in this declaration further demonstrate that the negative results are due to expression and secretion defects in VEGF-2 (as taught in the opposed application) rather than variations in transfection efficiency (as suggested, wrongly, by various HGS declarants). Also, I have attempted to further articulate the benefit of using two different transfection-efficiency controls (β -galactosidase and luciferase) in OKA3 and again here in OKA4.

1.5 Although the experimental design reported in OKA3 and the experimental design of the present declaration are very similar, the protocol described below included an extra adjustment in immunoprecipitation sample sizes, to normalize for apparent modest experimental variation in transfection efficiency or other factors influencing the quantity of recombinant protein expression over time. (See paragraphs 4.1 - 5.5, below.) Other minor changes were made in the experimental protocol of the present declaration (e.g., times of incubations) due to time constraints and do not affect the results of the types of experiments performed and described herein. The experiments described herein provide further evidence that cells cannot express and secrete VEGF-2 as taught in the opposed application. On this point, the results were the same as the data already presented in OKA1, OKA2, and OKA3, further confirming that these declarations were accurate and were experimentally sound.

II. EXPERIMENTAL PROCEDURE

A. Cells and Plasmids

2.1 Results reported in OKA2 revealed that COS and 293T cells were equally appropriate cell lines for analyzing VEGF-2 protein expression and secretion. For these experiments, 293T cells were grown in DMEM supplemented with 10 % fetal bovine serum, glutamine and penicillin/streptomycin.

2.2 The polymerase chain reaction (PCR) was employed to construct a cDNA fragment that corresponded to amino acids 70 to 419 of prepro-VEGF-C. For the purpose of these experiments (directed to assessing transfection efficiency and protein expression at various time points) the cDNA fragment encoding amino acid residues 70 to 419 of prepro-VEGF-C corresponds appropriately with the cDNA encoding the full length sequence of the VEGF-2 polypeptide described in the opposition application. Nucleotides 559 to 1608 of the VEGF-C cDNA (Reported in Document D70, Joukov et. al. 1996, GenBank accession number X94216) were PCR amplified with the primers 5'-CGCGGATCCATGACTGTACTCTACCCA-3' containing a BamHI site and 5'-CGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGG-GTACTCGAGGCTCATTGTGGTCT-3' containing a XbaI site, HA-tag, a stop codon and a XbaI site and cloned into pcDNA1(Amp)-vector (Invitrogen). The resultant vector was designated as VEGF-2(HGS)/pcDNA1.

2.3 An expression vector was also constructed that contained the full length (419 codons) VEGF-C sequence (OKA2 at 3.3.3) for use as a positive control in the expression and secretion analyses. The resultant vector was designated as VEGF-C/pcDNA1.

B. Transfection and time course:

2.4 A principle criticism alleged by the HGS experts was that my OKA2 declaration failed to include transfection efficiency data (ANH2 at 1.5; ASA2 at 24). Thus, for these new experiments, two separate expression vectors, pRL expressing Renilla Luciferase (Promega) and pCMV- β -gal expressing beta-galactosidase under CMV promoter, were used as transfection controls.

2.5 The other principle criticism of the procedures reported in my OKA2 declaration was regarding the lack of time points in the expression analyses (ANH2 at 1.5; ASA2

at 25). To address this concern, three different time points were tested in the new experiments. In particular, the 293T cells were split 1:6 and fresh medium was changed 19 hours thereafter. Three hours after medium change, VEGF-2(HGS)/pcDNA1, VEGF-C/pcDNA1, or empty vector were co-transfected with either pRL (three plates with each combination in a 16:1 ratio), or pCMV- κ -gal (one plate with each combination in a 1:1 ratio), using FuGENE Transfection Reagent (Roche). The conditioned media and the cells were harvested 24 hours, 48 hours, or 74 hours after the transfection for the purpose of evaluating protein expression and secretion at these different time points. Either twenty-four hours (for time points 48h and 74h) or eight hours (for time point 24h) prior to harvesting, the cells were washed twice with PBS and changed to 3 ml of MEM medium containing 100 μ Ci/ml 35 S-methionine and 35 S-cysteine (Promix, Amersham) for metabolic labeling of proteins synthesized by the cells¹. At the indicated time points the conditioned media was harvested and cleared by centrifugation. The cells were trypsinized, washed twice with PBS and lysed in 1x passive lysis buffer from the Dual-Luciferase Reporter Assay System (Promega).

C. Beta-galactosidase staining:

2.6 The cells were washed twice with PBS, fixed with 0.05% glutaraldehyde in PBS for 15 minutes at room temperature, washed three times with PBS, and stained over night with 2.5 mg/ml α -Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) at 37°C. The beta galactosidase data provided evidence of transfection efficiency.

D. Luciferase assay:

2.7 The protein concentrations of the cell lysates were determined by using the BCA Protein Assay (Pierce). To quantify expression of recombinant protein in transfected cells, luciferase activity in cell lysates was measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase data provided evidence of transfection efficiency and recombinant protein expression efficiency at various time points.

¹ See explanation in OKA2 at 3.4.2

E. Immunoprecipitation:

2.8 Immunoprecipitation experiments were conducted to identify the presence of VEGF-C or VEGF-2 polypeptides in the conditioned media from the cells after the indicated incubation times.

2.9 For immunoprecipitation, aliquots of each conditioned medium were supplemented with BSA and Tween 20 to final concentrations of 0.5% and 0.02%, respectively. The different VEGF-2 or VEGF-C peptides were immunoprecipitated with polyclonal antibodies raised against a synthetic peptide corresponding to amino acid residues 104-120 of the VEGF-C prepropeptide (Antisera 882, reported in Document D71, Joukov et al., 1997) at 4 °C overnight. This peptide is present in the secreted form of VEGF-C, and the opposed application teaches that it should be present in mature VEGF-2 as well. Thus, antisera raised against this peptide should recognize VEGF-2 or VEGF-C polypeptides produced by the cells.

2.10 The immunocomplexes of secreted polypeptides bound to antisera 882 were precipitated with protein A-Sepharose for 2 hours and washed 2 times with 1X binding buffer (0.5% BSA, 0.02% Tween20 in PBS) and once with PBS at 4°C. The proteins were analyzed by SDS-PAGE in a 12 % gel under reducing conditions.

III. EXPERIMENTAL RESULTS AND ANALYSIS**A. Beta-galactosidase and transfection efficiency**

3.1 In order to analyze transfection efficiency, the constructs of interest (VEGF-2(HGS)/pcDNA1, VEGF-C/pcDNA1, or the mock vector) were co-transfected into 293T cells with a plasmid encoding beta-galactosidase. Beta-galactosidase is an enzyme that causes production of a colorometric product, under the assay conditions used. In particular, successful transfection with the beta-galactosidase plasmid is observable as a colored colony on a culture plate. The presence and percentage of colored cells that are observable following the transfection experiments provides a measurement of relative transfection efficiency for the different transfections. HGS's declarant, Dr. Power, chose a beta galactosidase for her transfection efficiency control in her ASP2 declaration (See ASP2 at 20 and 24).

3.2 Ludwig Institute asked that I provide actual data in this declaration for the transfection efficiency study. Accordingly, I have included photographic results of the beta-

galactosidase staining as Figure 1B attached hereto (see Exhibit KA-1 hereto). The figure represents photographs of culture plates following plating, transfection, and growth of the cells. Dark colored "spots" represent colonies formed from cells that were successfully transfected with the beta galactosidase vector and that are therefore expressing beta-galactosidase and appear dark blue under the assays conditions used. White or colorless cells, on the other hand, are not expressing the beta-galactosidase, and are scored as negative with respect to transfection.

3.3. The photographs in Figure 1B reveal that each of the transfections were very successful (numerous dark colored colonies were observed on all of the plates), and that the percentage of transfected cells were comparable in each of the three transfection groups (i.e., VEGF-2, VEGF-C, and mock). This data is sufficient to conclude that transfection efficiency is not confounding the results of my comparative expression studies, thus dispelling one of HGS's main criticisms of my OKA2 declaration.

B. Luciferase and Protein expression

4.1 The luciferase experiments provide a supplemental control for transfection efficiency that complements the beta-galactosidase study, and also provide an indication of the level of recombinant protein expression in transfected cells. The luciferase study was comparable to the beta-galactosidase study in the sense that it involved co-transfection of the constructs of interest (VEGF-2 or VEGF-C plasmid) with a second construct, the presence of which was measurable by a visualization assay. Because the co-transfected luciferase plasmid encodes an enzyme that causes production of light waves under the assay conditions that were used, the measurements of light units provides an indication that the transfections were successful. However, the luciferase assay differs from the beta-galactosidase assay because it quantitatively measures recombinant protein from transfection plates, whereas the beta galactosidase assay only measures the number of transfected versus non-transfected colonies, and does not provide a quantitative measurement of the amount of recombinant protein that the cells are producing.

4.2 The VEGF-2(HGS)/pcDNA1, VEGF-C/pcDNA1, or the mock vector were co-transfected into 293T cells with the plasmid encoding the Renilla luciferase gene as described above. At the datapoints selected for the experiment (24, 48, and 74 hours after transfection) conditioned medium was collected to assay for the secretion of VEGF-2 or VEGF.

C by the cells, and cell lysates were also analyzed to quantify the luciferase activity in the cells at the same time points.

4.3 The results of the luciferase assay are presented as a bar graph in Figure 1A (see Exhibit KA-1 hereto), showing relative light units per microgram of protein for each of the three transfection groups at each of the time points specified. Since the luciferase assay is a measure of recombinant protein expression, it provides a second indication that both the VEGF-2 and the VEGF-C transfections were successful, because all groups showed luciferase activity at all time points. The fact that the relative levels of luciferase (in the VEGF-2 versus VEGF-C cultures) varies somewhat with time reflects the fact that the luciferase measurements are quantitative for the amount of recombinant, active luciferase protein present, rather than quantitative for percentage of cells successfully transfected. At all time points studied, the luciferase measurements in the VEGF-2 and VEGF-C plates were of the same order of magnitude.

C. Analysis of VEGF-2 and VEGF-C expression and secretion.

5.1 At the outset, it is my opinion that the differences in luciferase measurements between VEGF-2 and VEGF-C plates do not reflect a variable involving transfection efficiency that could account for the differences in VEGF-2 versus VEGF-C protein expression and secretion that I reported in all three of my previous declarations. In fact, the opposite is true. The luciferase data serves to validate the experimental design. The luciferase measurements were the same order of magnitude at each time point and indicate successful transfection of cells in both the VEGF-2 and the VEGF-C co-transfection experiments. The luciferase activity was abundant and measurable for both the VEGF-2 and VEGF-C co-transfections, whereas in the immunoprecipitation experiments, VEGF-2 has always been unmeasurable, while VEGF-C has always been easily measured. Thus, the transfection and cell culturing techniques are all sound. The "problem" is that the cells cannot express and secrete the VEGF-2 encoded by the VEGF-2 cDNA as taught in the opposed application.

5.2 Even so, for this declaration, I adjusted the immunoprecipitation experiments for the benefit of the VEGF-2 transfections, based on the luciferase data. Specifically, I presumed that the luciferase measurements provided an indication of the recombinant protein making capacity of the transfected cells. Based on this assumption, the

VEGF-C cells were making more recombinant protein than the VEGF-2 cells in these particular transfections. To compensate for the apparent difference, I used larger volumes of conditioned media from the VEGF-2 cells than from the VEGF-C cells for the immunoprecipitation. The volumes selected were as follows:

Culture Period	Volume of CM from cells transfected with VEGF-2(HGS)/pcDNA1	Volume of CM from cells transfected with VEGF-C/pcDNA1	Volume of CM from mock transfected cells
24 hours	690 microliters	450 microliters	1000 microliters
48 hours	235 microliters	125 microliters	1000 microliters
74 hours	265 microliters	180 microliters	1000 microliters

5.3. The relative amounts of conditioned media used were inversely proportional to the luciferase measurements. In each instance, more conditioned media from the VEGF-2 cells was used than from the VEGF-C cells to compensate for the lower production of recombinant (luciferase) protein in these cells. (If the VEGF-2 cells were secreting less protein per microliter of conditioned media, the use of larger sample sizes would compensate.)

5.4 No VEGF-2 protein was detected in the conditioned media from the cells transfected with the VEGF-2(HGS)/pcDNA1 construct at any of the time points tested, over a time period of 74 hours (Figure 2, Lanes 1, 4 and 7; see Exhibit KA-2 hereto. The only bands visible in the VEGF-2 lanes of the gel are also visible in the "mock" lanes that have no VEGF-2 construct). In contrast, VEGF-C protein was effectively expressed and secreted by cells transfected with a vector encoding the full length VEGF-C. The different forms of VEGF-C immunoprecipitated from the conditioned medium correspond to partially and fully processed forms of VEGF-C (Figure 2, Lanes 2, 5 and 8. See bands corresponding approximately to the 30, 21.5, and 14.3 size markers). These results are in agreement with the results reported in OKA1, OKA2 and OKA3, and provide still further evidence that VEGF-2 as taught in the opposed application cannot be expressed and secreted by cells.

5.5 As Figure 2 shows, cells do not express and secrete VEGF-2, no matter what time point is used to terminate the experiment. Using extra conditioned media from the VEGF-2 cells fails to change the negative result. Expression of VEGF-C polypeptides, which serves as a positive control, was visible at all time points studied. VEGF-C expression was already visible at 24 hours and was strongly visible at 48 hours and 74 hours.

5.5 The extra experiments that I ran for OKA3 and this declaration demonstrate that all of HGS's criticisms of my earlier experiments were meritless. The protein expression and secretion data reported in OKA3 and the present declaration is essentially identical, indicating that transfection efficiency, time course, and any other miscellaneous factors causing minor fluctuations in recombinant protein production from one experiment to another do not alter the end result. The data reported herein confirms the conclusions of OKA1, OKA2, and OKA3, namely, that cells cannot express and secrete VEGF-2 as taught in the opposed application.

IV. CONCLUDING REMARKS

6.1 The protein expression and secretion studies I report herein were designed to address any criticisms made by HGS with regard to experimental design credibility. The results demonstrate several key points. First, VEGF-2 as taught in the opposed application cannot be expressed and secreted by cells. The data clearly establishes the failure of VEGF-2 to be expressed and secreted at multiple time points over a period of 74 hours (Each of these time points was sufficient to observe expression and secretion of the VEGF-C positive control run under the same experimental conditions). Second, transfection efficiency experiments reported herein rule out the possibility that absence of expression of VEGF-2 was due to insufficient amounts of the VEGF-2 expression vector being introduced into the cells. Even if the minor variations in transfection efficiency could contribute to variations in protein expression and secretion, this phenomenon was accounted for in the immunoprecipitation experiments reported herein by increasing the volumes of the VEGF-2 conditioned medium that were used in the immunoprecipitations. Thus, transfection efficiency, expression and secretion time course, and all other meaningful variables have all been accounted for in this declaration. This declaration,

and my three earlier declarations, all demonstrate that cells cannot express and secrete VEGF-2 as taught in the opposed application.

AND I MAKE this solemn declaration by virtue of the Statutory Declarations Act 1959, and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true in every particular.

DECLARED at ...Helsinki, Finland.....

This 14 day of August, 2002

Kari Alitalo

BEFORE ME

(Signature of Notary Public)
HANS SEVELLUS
Notary Public
14.08.2002



AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent
Application Serial No. 696764 by Human
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by
Ludwig Institute for Cancer Research

THIS IS Exhibit KA-1

referred to in the Statutory Declaration
of Kari Alitalo
made before me

DATED this 14th Day of August 2002

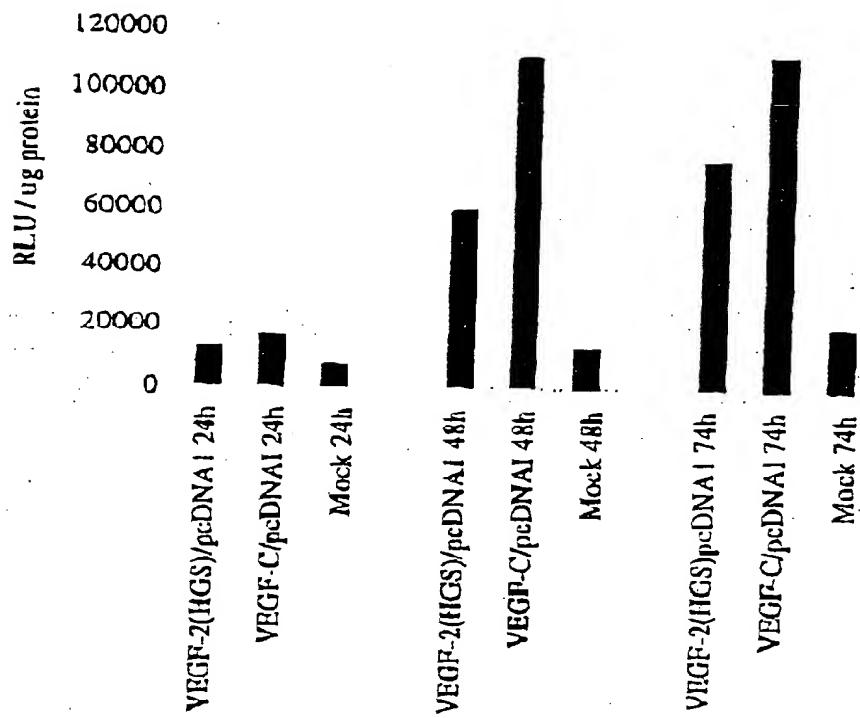


(Signature of Notary Public)
HANS SEVELIUS
Notary Public

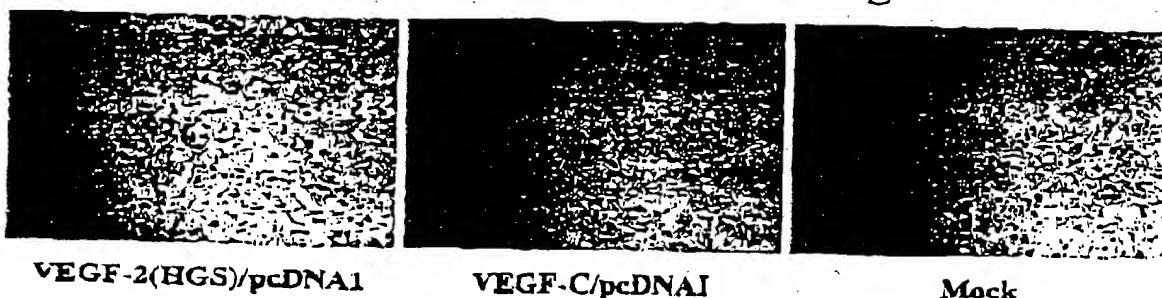


A

Renilla Luciferase Assay

**B**

Beta-galactosidase Staining



AUSTRALIA*Patents Act 1990*

IN THE MATTER OF Australian Patent
Application Serial No. 696764 by Human
Genome Sciences, Inc.

-and-

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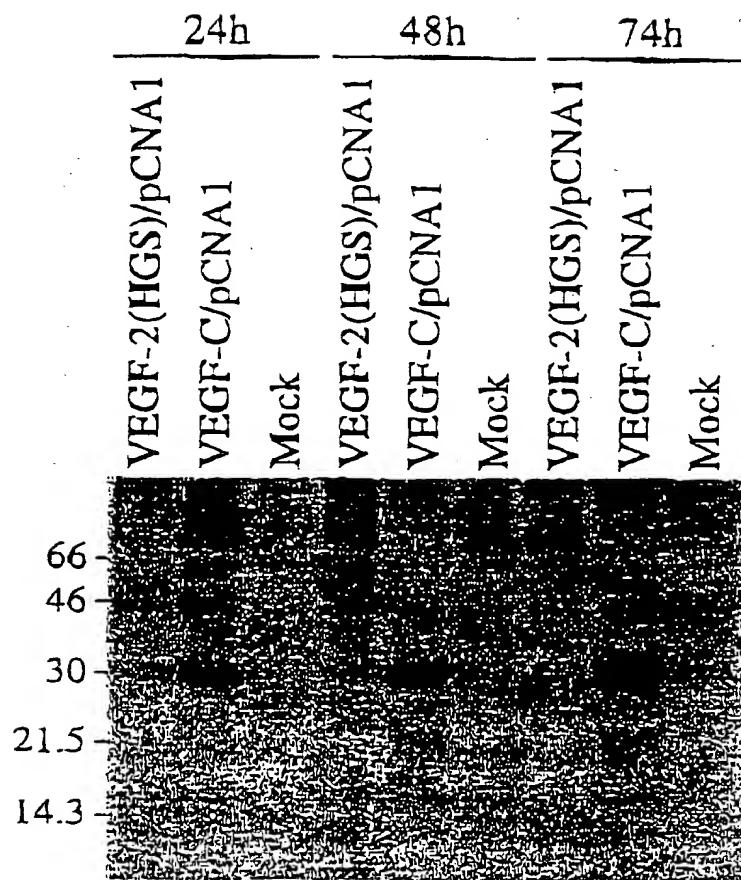
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referred to in the Statutory Declaration
of Kari Alitalo
made before me

DATED this 14th Day of August 2002


(Signature of Notary Public)
HANS SEVELIUS
Notary Public





Figure